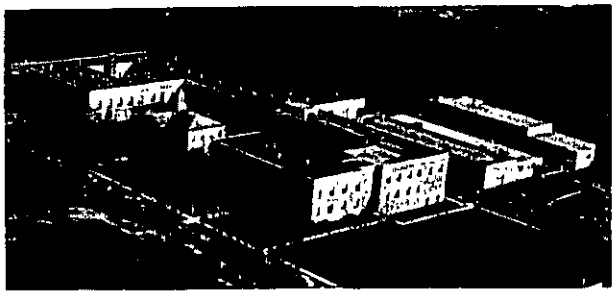

THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

THE GROWTH OF ISOLATED ASPEN TISSUE OF TRIPLOID ORIGIN

Project 2351

Report One

A Progress Report

to

PIONEERING RESEARCH COMMITTEE

January 21, 1963

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SUMMARY

Actively growing aspen tissue can be maintained on a medium containing major elements, trace elements, sucrose, coconut milk, and naphthaleneacetic acid. Satisfactory growth was obtained when medium containing naphthaleneacetic acid, at concentrations from 0.5 to 8.0 p.p.m., was supplemented with 100 p.p.m. of casamino acids or malt extract. Cultures may be maintained on agar or in liquid medium. The rate of growth of isolated tissue is increased when the temperature is increased from 25° C to 30° C. Aspen tissue may contain areas of red, yellow or green pigmentation. The dominant pigmentation present can be changed by manipulation of the medium and enviromental conditions.

*This report will be submitted for publication in Forest Science, pending approval by the Pioneering Research Committee.

INTRODUCTION

The importance of the *in vitro* culture of isolated plant tissues has been recognized as a technique useful in the study of various physiological processes since 1902 (1). Further development of methods by White (2) and others for the cultivation of cells for indefinite periods made detailed nutritional and developmental investigations possible. The study of the growth of tissues from woody species of known origin may provide techniques useful in developing propagation methods for the production of genetically uniform materials. Isolated tissue may also prove to be an important tool in the study of the growth and disease resistance of forest species.

The present study was designed to determine media capable of supporting active growth of aspen tissue for an indefinite period. This investigation was not intended to be a study of the possible media components but was designed to provide data necessary for the selection of media and environmental conditions which will be used to maintain tissue for use in subsequent investigations. No attempt was made to select the optimum conditions or medium for the growth of aspen tissue. This study is the first of a series of investigations which will involve the growth and differentiation of isolated aspen tissue, the chemical composition of the tissue and the *in vitro* production of inhibitory materials active against a number of bacteria and fungi.

MATERIALS AND METHODS

On November 5, 1961, root samples were removed from a triploid aspen (Populus tremuloides) growing in an experimental plot in Greenville, Wisconsin. Roots with an approximate diameter of one inch were cut into four-inch lengths and placed in a sand–vermiculite mixture in a greenhouse. Small root sprouts were transferred to pots and on December 11, 1961 the leaves were removed from materials which were approximately two inches tall. The stems were cut into internode sections and surface sterilized by immersion for ten minutes in a 5.25% solution of sodium hypochlorite, containing a small amount of Tween 20. The materials were rinsed in sterile distilled water and cut into sections from 5 to 10 mm. in length. Sections were transferred, under aseptic conditions to a basal medium containing nutrients (White) (3), trace elements (Nitsch) (4) 2% sucrose and 10% coconut milk. The coconut milk was heated to 60°C., as suggested by Tulecke (5). Callus proliferation was obtained in the dark at 24-26°C. and tissue from the approximate cambial area was isolated on December 26, 1961. This isolated tissue, after a number of transfers and considerable growth, was used as the experimental material. The materials and methods of tissue isolation are illustrated in Fig. 1.

In general, tissue grown in the dark on agar media forms compact masses which contain brown relatively slow growing areas and white areas of active growth. Actively-growing tissue was removed and cut into small sections weighing approximately 5 mg. each. The weight of the tissue used in each experiment was determined by weighing sterile, tared 10-ml. Erlenmeyer flasks containing a few drops of basal medium and the experimental material. Ten pieces of tissue, which had been selected for uniformity were placed in Petri dishes, containing the appropriate medium and allowed to grow for three weeks in the dark at 24-26°C. At

the end of each experiment the tissue was removed from the agar, reweighed and the per cent increase was calculated. Considerable variation in the rate of growth was encountered and as a result all experiments were repeated at least twice.

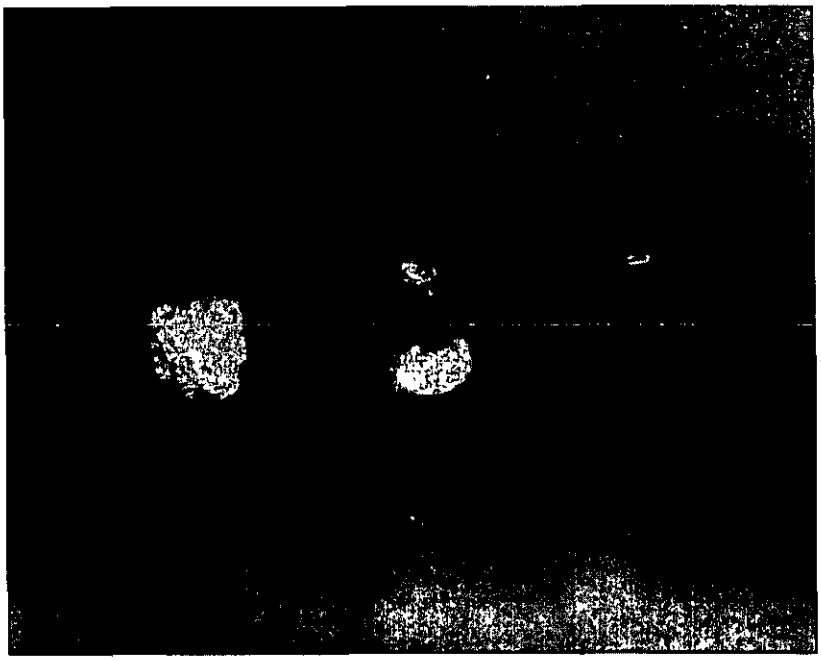

Figure 1. Method of Tissue Isolation
Right—Internode Section of the Stem
Center—Section Showing Callus Formation After Approximately Twenty Days of Growth
Left—Isolated Tissue After Additional Growth

RESULTS

EFFECT OF VARIOUS SUPPLEMENTS ON THE RATE OF GROWTH

In the first series of experiments the basal medium (BM) contained one of the following supplements: 200 p.p.m. yeast extract (YE, Balt. Biol. Lab.), 200 p.p.m. casamino acids (CA, Difco) or 200 p.p.m. malt extract (ME, Balt. Biol. Lab.) and 0.5 p.p.m. indoleacetic acid (IAA) or naphthaleneacetic acid (NAA). In all experiments the various supplements and combinations of supplements were added to the basal medium and autoclaved for 20 minutes at 15 p.s.i. The results (Table I, Column 1) were based on the per cent increase in fresh weight obtained on basal medium. The addition of a low concentration of NAA was responsible for a marked increase in the amount of growth. Conversely, the addition of IAA, YE, CA, or ME had essentially no effect on the growth of aspen tissue. It is, however, possible that the addition of these supplements in concentrations other than those used in this series may result in a stimulation in the rate of growth. In the next series of experiments, supplements, as listed previously, were added to BM containing 0.5 p.p.m. IAA. The addition of these supplements (Table I, Column 2) did not result in a significant stimulation in the rate of growth of the isolated tissue.

In the final series of experiments, (Table I, Column 3) supplements were added to BM containing 0.5 p.p.m. NAA. The observations indicated that ME and CA may be slightly stimulatory in terms of fresh weight production.

The next phase of the experimental program included an investigation of the growth of tissue supplied with various levels of the supplements. Malt extract and CA were added, in concentrations of 100, 200, 400, 800 or 1600 p.p.m., to BM

containing 0.5 p.p.m. NAA. The addition of 100 p.p.m. ME or CA resulted in a slight increase in the rate of growth, as compared with the rate of growth on BM containing 0.5 p.p.m. NAA and 200 p.p.m. of the supplement. Increasing concentrations, 0.5, 1, 2, 4 or 8 p.p.m., of NAA also resulted in slight increases in the rate of growth of the isolated aspen tissue.

TABLE I

THE EFFECT OF VARIOUS BASAL MEDIUM SUPPLEMENTS ON THE GROWTH[a] OF ISOLATED ASPEN TISSUE

Supplement Added[b]	Percent of Control		
	BM	BM+IAA	BM+NAA
None (control)	100	100	100
YE	84	70	115
CA	110	104	139
ME	102	80	138
IAA	110	--	--
NAA	254	--	--

[a] cultures grown in the dark on agar medium at 24-26°C.
[b] YE- 200 p.p.m. yeast extract; CA- 200 p.p.m. casamino acids; ME- 200 p.p.m. malt extract; IAA- 0.5 p.p.m. indoleacetic acid; NAA- 0.5 p.p.m. naphthaleneacetic acid; BM- basal medium

The results from these studies indicate that basal medium supplemented with 0.5 to 8 p.p.m. NAA alone or in combination 100 p.p.m. ME or 100 p.p.m. CA can be used for the maintainence of actively growing aspen tissue.

GROWTH RATE (Fig. 2)

Fifteen Petri dishes, each containing ten pieces of tissue (reps) and BM supplemented with 0.5 p.p.m. NAA, were placed in the dark at 24-26°C. At the beginning of the experiment and after each four-day period ten pieces of tissue

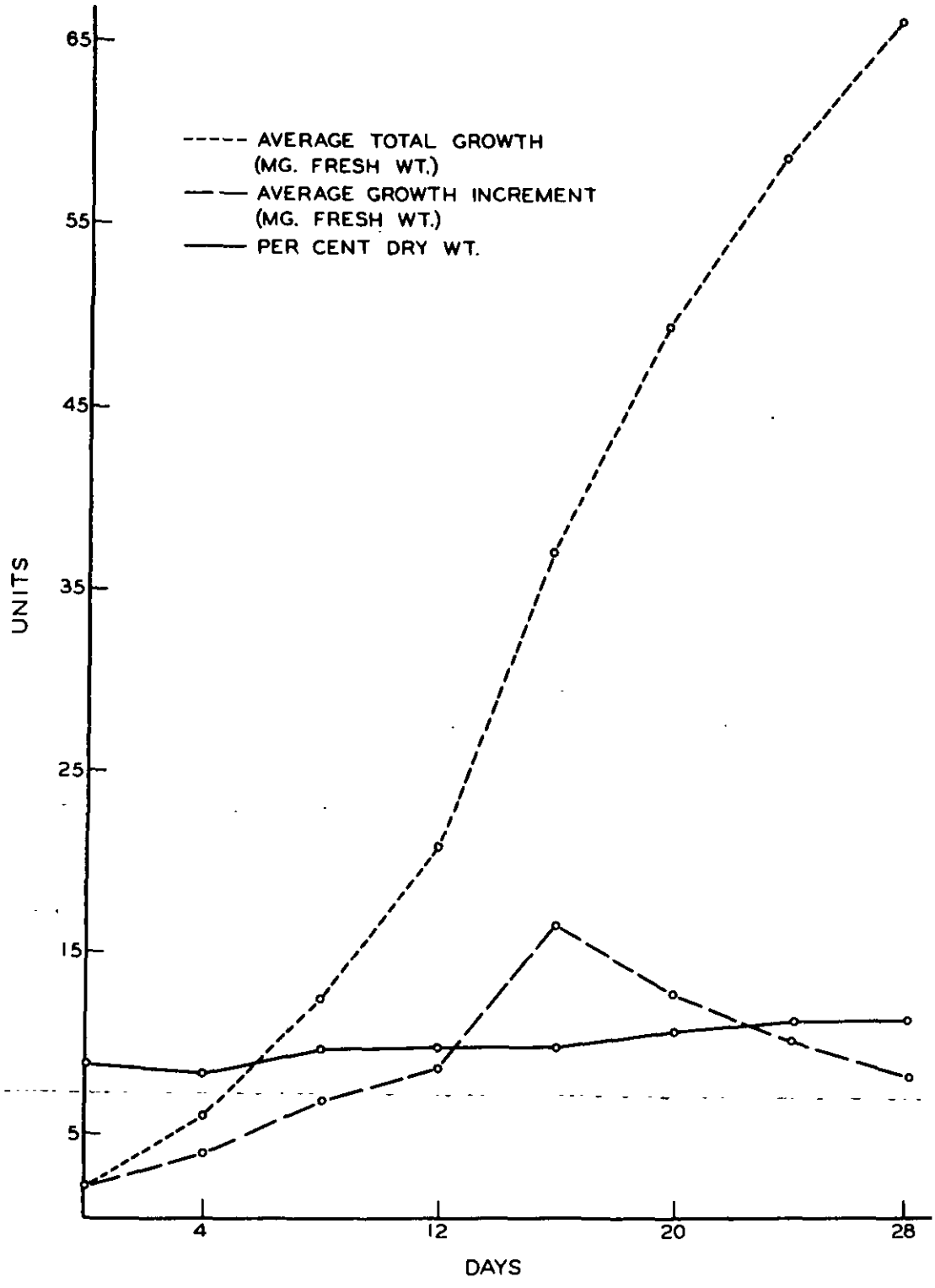

Figure 2. Growth Rate of Aspen Tissue Grown in the Dark at 25-26°C., on a Basal Agar Medium Containing 0.5 p.p.m. Naphthaleneacetic Acid

were sampled, dried in the oven at 75°C. and used in the dry weight determinations. The remaining tissue, in groups of ten pieces each, was weighed in order to obtain the fresh weight and allowed to grow for a period of four days. This procedure was repeated throughout the 28-day period of growth. The tissue was maintained in an active state of growth throughout the experiment. A comparison of the rate of growth of reps, however, revealed considerable variation. Certain variables, such as physiological differences, injury due to cutting or differences in the surface area may account, at least partially, for the observed variation between apparently uniform tissues. In this experiment, growth in terms of increased fresh weight was more rapid after 12 days with the greatest amount of materials being incorporated between the twelfth and sixteenth days. The per cent dry weight remained relatively constant throughout the experiment. This proportion of approximately ten parts fresh weight to one part solid material means that the measurement of fresh weight can be used in the approximation of dry weight.

INFLUENCE OF TEMPERATURE ON THE RATE OF GROWTH

Petri dishes, each containing ten pieces of tissue and BM supplemented with 0.5 p.p.m. NAA were placed in incubators and allowed to grow for three weeks. The temperatures used were 25-26, 30-31, and 35-36°C. The per cent increase in the fresh weight of tissue grown at 25-26°C. was used as the control. It was found that the rate of growth decreased slightly at 35-36°C. while a significant increase, approximately twice the control growth rate, was obtained at 30-31°C. In general, satisfactory growth of aspen tissue was evident at temperatures ranging from 25-31°C.

THE INFLUENCE OF VARIOUS FACTORS ON THE GROWTH AND FORM OF ASPEN TISSUE

Isolated aspen tissue grown on an agar medium (BM supplemented with 0.5 p.p.m. NAA) in the dark at temperatures of 25-31°C. is composed of compact masses of tissue containing white or cream-colored areas of active growth and brown areas of relatively slow growth (Fig. 3). Cultures grown on a shaker (rotary, 120 r.p.m.) in liquid medium (BM supplemented with 0.5 p.p.m. NAA) in the dark at 29-31°C. result in compact masses of tissue which are bright yellow in color (Fig. 4). This tissue is actively growing with approximately 2700% increase in fresh weight in a period of three weeks and is very uniform in appearance. Tissue grown on an agar medium (BM containing 0.5 p.p.m. NAA) at room temperature under continuous illumination of approximately 250 foot candles will develop a bright red pigmentation while tissue grown under the same conditions will develop distinct areas of green pigmentation, in addition to areas of red color, when the sucrose level is reduced to 0.25%.

The differentiation of roots was observed when aspen tissue was grown in the dark on an agar medium containing various supplements (Fig. 5). Root differentiation was not observed in the light or on liquid medium and there seems to be no direct correlation between the presence of certain supplements, as used in this study and the appearance of roots. Shoot differentiation was not induced by the conditions or media employed in this study. Further investigations will be required in order to critically evaluate the factors involved in root and shoot differentiation.

Figure 3. Typical Tissue Grown on an Agar Basal Medium Supplemented with 0.5 p.p.m. Naphthaleneacetic Acid. This Tissue was Grown in the Dark at 25-27°C.

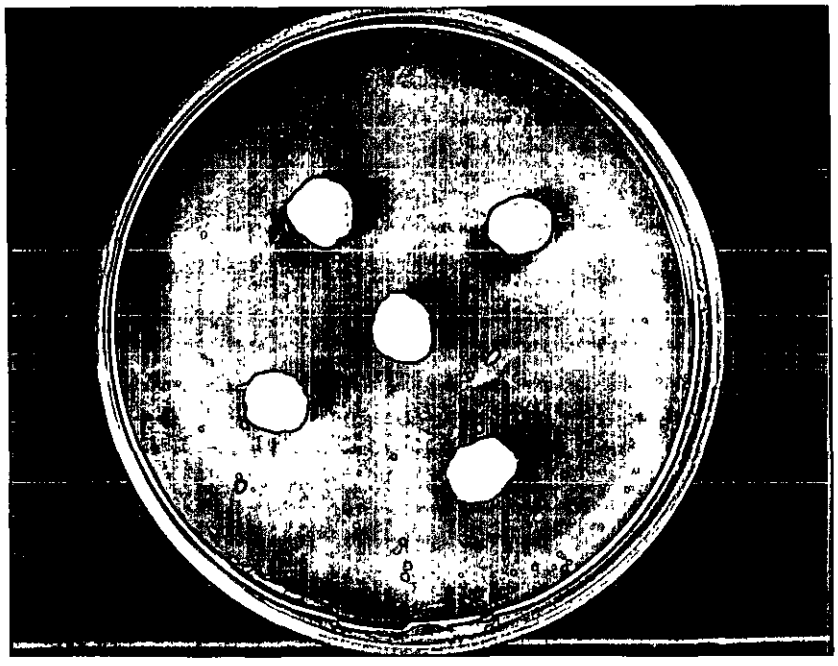

Figure 4. Typical Tissue Grown in Liquid Basal Medium Supplemented with 0.5 p.p.m. Naphthaleneacetic Acid. This Tissue Was Grown in the Dark on a Rotary Shaker (Approximately 120 r.p.m.) at 29-31°C.

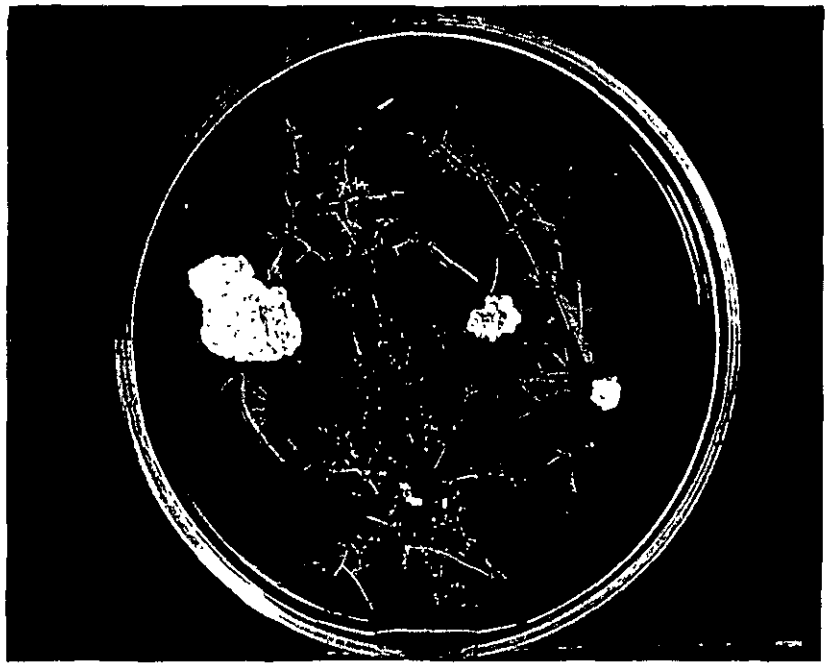

Figure 5. Isolated Aspen Tissue Which Has Differentiated to Form a Branched Root System. This Tissue Was Grown on Agar Basal Medium, Supplemented with 0.5 p.p.m. Naphthaleneacetic Acid, in the Dark at 25-27°C.

FUTURE PLANS

The next phase of the program will be directed toward the demonstration of an "antibiotic" substance which is produced by isolated aspen tissue. The work presently in progress concerns the determination of a spectrum of activity using a number of bacteria and fungi. After completion of preliminary investigations, attempts will be made to characterize the type of inhibition and to determine the nature of the inhibitory material and its occurrence in various members of the genus Populus.

Attempts to obtain isolated Haplopappus tissue were unsuccessful. After considerable searching, a source of seeds was found and the material is

presently growing in the greenhouse. The plants have reached a suitable size and callus tissue should be isolated in the near future. This tissue will be used as the experimental material in studies dealing with the strand separation of nuclear material. Preliminary investigations concerning high-temperature ultracentrifugation methods of strand separation and recombination will be conducted by Dr. Elwood O. Dillingham of the Institute staff. This work will be directed toward perfecting methods of deoxyribonucleic acid extraction and strand separation using bacteria.

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